

## Genetic structure and introgression in riparian populations of *Populus alba* L.

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### Abstract

White poplar (*Populus alba*) is a widespread species of the northern hemisphere. Introgressed populations or hybrid zones with the related species of the European aspen (*Populus tremula*) have been suggested as potential venues for the identification of functionally important variation for germplasm conservation, restoration efforts and tree breeding. Data on the genetic diversity and structure of introgressed *P. alba* are available only for sympatric populations from central Europe. Here, clonality, introgression and spatial genetic patterns were evaluated in three riparian populations of *P. alba* along the Ticino, Paglia-Tevere and Cesano river drainages in Italy. Samples of all three populations were typed for five nuclear microsatellite markers and 137 polymorphic amplified fragment length polymorphisms. Microsatellite-based inbreeding co-efficients ( $F_{IS}$ ) were significantly positive in all three populations. Genetic diversity was consistently highest in Ticino, the population with the highest level of introgression from *P. tremula*. Population differentiation ( $F_{ST}$ ) was low between the Ticino valley in northern Italy and the Cesano valley in central Italy and between the central Italian populations of Cesano and Paglia-Tevere, consistent with a role of the Appenine mountain range as a barrier to gene flow between adjacent drainage areas. Introgression was not the primary determinant of within-population spatial genetic structure (SGS) in the studied populations.

**Keywords:** *Populus*, hybridisation, introgression, microsatellite, amplified fragment-length polymorphism

### Introduction

Many plants and some animal species have weak reproductive barriers allowing gene flow to occur not only between populations of the same species but also between morphologically distinct species (Stebbins 1959; Arnold 1997). This process is known to conservation geneticists as introgression or continued hybridisation (Wolf et al. 2001; Butcher et al. 2005; Oliveira et al. 2008). It can be of serious concern because it can trigger the evolution of invasive taxa (Ellstrand & Schierenbeck 2000) or lead to the extinction of taxa with small effective population sizes ( $N_e$ ) when they are "swamped" by gene flow from more abundant species (Wolf et al. 2001; Ferdy & Austerlitz 2002).

Other authors have stressed the potentially positive roles of hybridisation in evolution. For instance, genetic recombination between previously divergent species may also lead to the origin of new hybrid species (Rieseberg et al. 2003) or to the transfer of ecologically important, adaptive traits from one species into the genetic background of another (Arnold 1997; Barton 2001). Thus, it has sometimes been suggested that natural hybrid zones should be protected as localities where ecologically relevant variability can evolve and be maintained (Cozzolino et al. 2006).

A third and different view of natural hybrid zones is currently emerging in conservation genetics. It is fuelled by rapid progress in genomic technologies (Noor & Feder 2006) and by the observation that

the increased variability often found in natural hybrid populations may be used to identify adaptively important variation (Lexer et al. 2004; Buerkle & Lexer 2008). Since hybrid zones often contain a large number of recombinant individuals, they potentially allow genetic mapping of phenotypes through admixture Linkage Disequilibrium (admixture LD) (Briscoe et al. 1994; Rieseberg et al. 1999; Buerkle & Lexer 2008). This adds a completely different aspect to the conservation status of hybrids, as it implies that hybrid zones may sometimes be invaluable genetic resources for the genetic characterisation of adaptive traits, which is an important aspect of forest conservation genetics (Lexer et al. 2004) and restoration ecology (Hufford & Mazer 2003). Pioneering studies indicate that phenotypic traits can indeed be mapped to the genome in natural hybrid zones of plants, e.g. pollen sterility as a species isolation factor in *Helianthus* hybrid zones (Rieseberg et al. 1999). Nevertheless, the potential to identify and map ecologically important, adaptive traits in hybrid zones has been little explored (Buerkle & Lexer 2008).

Recently, natural hybrid zones between the two European forest tree species *P. alba* and *P. tremula* have been suggested as suitable genetic resources to identify and map genetic variation associated with adaptively important traits (Lexer et al. 2005). The two species are ecologically divergent (*P. alba*: floodplain pioneer, *P. tremula*: upland pioneer) (Adler et al. 1994), and some of the traits involved in these ecological differences are known (Karrenberg et al. 2002; Lexer et al. 2004). Genetic marker-based studies indicate that natural hybrids between the two species exist in several European river drainage systems, including recombinant backcrosses towards *P. alba* (Bartha 1991; Rajora & Dancik 1992; Fossati et al. 2004; Lexer et al. 2005). However, so far only one of these populations has been characterised in detail with respect to hybrid abundance, genomic composition and fine-scale spatial genetic structure (SGS), namely the central European hybrid zone in the Danube valley in Austria (Lexer et al. 2005; van Loo et al. 2008). In order to make full use of these natural genetic resources for the identification and study of adaptive variation, it is necessary to search for “replicate” hybrid zones for gene mapping. Additional hybrid populations would facilitate the identification of genomic regions, or loci that depart from neutral expectations not only in one but in several localities, thus providing the necessary replication for the genetic work (Buerkle & Rieseberg 2001).

Here, we characterised introgressed Italian populations of *P. alba* to verify their suitability for this purpose, and asked the following questions regarding the conservation genetics of Italian populations of *P. alba*: (1) How similar, or different, are genetic

structures and levels of variability in Italian populations compared to other populations in central Europe, and what are the effects of introgression from *P. tremula* on genetic variability in Italian *P. alba* populations? (2) How strong is the fine-scale SGS within the Italian populations, and to what extent is it affected by introgression? (3) What are the likely implications of introgression for the conservation of “neutral” genetic diversity in riparian populations in Italy? (4) Is any of the Italian populations likely to be suitable for identifying functionally important variation by using the suite of methodologies of admixture mapping?

## Materials and methods

### *Plant material and study sites*

In total, 154 *Populus alba* L.-like trees were sampled randomly within three populations along the Ticino, Paglia-Tevere and Cesano rivers in Italy (Figure 1). The Ticino sample ( $N = 49$ ) was collected in the lowland floodplain forest of the “Lombard Park of the Ticino Valley” (<http://www.parks.it/parco.ticino.lombardo/Eindex.html>), covering a stretch of about 22 km of the river valley. The park was established in 1974 and is considered an important biodiversity resource for poplar and other plant species (Sartori 1985; Fossati et al. 2003, 2004). The other two riparian populations, Paglia-Tevere ( $N = 53$ ) and Cesano ( $N = 52$ ), are separated by a smaller distance (Figure 1), but are located on opposite slopes of the Apennine mountain range. The three populations differ in population demography and forest management. In Paglia-Tevere, trees are distributed uniformly in the riverine forest, whereas in Ticino and Cesano trees form clusters separated by short and large distances, respectively. Furthermore, the populations of Paglia-Tevere and Cesano are managed by man, whilst in Ticino human disturbance has been minimal since the park was founded.

Field collections in all three populations were carried out without taking into account tree morphology, as it is difficult to identify hybrids within *P. alba* populations on the basis of morphological characters alone (Adler et al. 1994). Hybrids and introgressants [*P. × canescens* (Aiton) Sm], carrying genetic material from the closely related *P. tremula* L., were subsequently identified in the laboratory using amplified fragment length polymorphism (AFLP) markers (see below). For each riparian population, groups of nearby individuals (minimum distance = 10 m) were collected in spots where poplar trees were present. This non-exhaustive type of sampling allows estimation of basic genetic diversity parameters and provides conservative estimates of clonality. It also facilitates



Figure 1. Map showing the three riparian populations of *P. alba* studied in northern Italy (Ticino) and central Italy (Cesano to the east and Paglia-Tevere to the west). The background shows the main biogeographical regions of Italy according to the EEA biogeographical map of Europe (2001), including the biogeographical subdivision due to the Apennine mountain range.

analysis of fine-scale SGS, because it yields a broad distribution of inter-individual geographic distances (Vekemans & Hardy 2004). The position of each single tree was recorded using a Global Positioning System (GPS) device. The age of most collected trees was estimated, on the basis of the trunk diameter, to be between 20 and 100 years.

#### *DNA extraction and microsatellite genotyping*

Total genomic DNA was extracted from 20 mg of dried leaves per tree using the DNeasy Plant Mini Kit (Qiagen, Milano, Italy). Five independent nuclear microsatellite loci (Table I) were used to obtain multi-locus genotypes. The microsatellites were developed by Van der Schoot et al. (2000) and Smulders et al. (2001) and are available at the following web site: <http://www.ornl.gov/sci/ipgc/>

[ssl\\_resource.htm](http://www.ornl.gov/sci/ipgc/ssl_resource.htm). In polymerase chain reactions (PCRs), a traditional two-primer approach (in which the forward primer was fluorescence-labelled) was utilised. For microsatellite analysis, PCR amplifications were performed in 15- $\mu$ l reactions containing 1–10 ng of DNA, 1 $\times$  PCR Green reaction buffer (Promega), 200  $\mu$ M of each dNTP, 0.75-U Taq polymerase (GoTaq, Promega-Italia), 1.5-mM MgCl<sub>2</sub> and 0.4  $\mu$ M of each primer. The PCR cycling conditions were as in Fossati et al. (2004). The forward primers were labelled with the fluorescent dyes FAM, HEX or TMR (MWG Biotech, Germany). The PCR products were separated by capillary electrophoresis, with a 400-bp size standard (Amersham, Uppsala, Sweden) using a MegaBACE automatic sequencer (Amersham, GE Healthcare). Alleles were sized using Fragment Profiler version 1.2 (Amersham, Uppsala, Sweden).

Table I. Characterisation of microsatellite markers at the genet level in three introgressed riparian populations of *P. alba* in Italy, including repeat type, number of individuals ( $N^*$ ), number of alleles ( $A$ ), allelic richness (AR), expected ( $H_E$ ) and observed ( $H_O$ ) heterozygosity and inbreeding coefficient ( $F_{IS}$ ). Overall statistics and standard deviations (SD) are given. Significant departures from Hardy-Weinberg equilibrium (HWE) are indicated by asterisks.

Population	Index		Locus					Overall	SD
	Marker name		<sup>a</sup> WPMS5	<sup>b</sup> WPMS14	<sup>b</sup> WPMS15	<sup>b</sup> WPMS18	<sup>b</sup> WPMS20		
Repeat type		di	tri	tri	tri	hexa			
Cesano	$N^*$	41	52	50	50	52	53	—	
	$A$	5	6	5	5	1	4.4	1.949	
	AR	5.000	5.954	4.969	4.640	1.000	4.313	1.916	
	$H_E$	0.590	0.510	0.684	0.531	0.000	0.463	0.267	
	$H_O$	0.415*	0.481	0.560*	0.580	0.000	0.407	0.237	
	$F_{IS}$	0.308	0.068	0.191	-0.083	NA	0.131	0.168	
Paglia	$N^*$	49	52	42	51	53	52	—	
	$A$	6	6	6	6	3	5.4	1.342	
	AR	5.833	5.871	6.000	5.962	2.989	5.331	1.311	
	$H_E$	0.715	0.538	0.761	0.725	0.175	0.583	0.244	
	$H_O$	0.837	0.385*	0.381*	0.666	0.132*	0.480	0.275	
	$F_{IS}$	-0.160	0.293	0.509	0.091	0.253	0.186	0.249	
Ticino	$N^*$	48	49	47	49	49	49	—	
	$A$	6	7	7	7	2	5.8	2.168	
	AR	5.997	6.967	6.985	6.999	2.000	5.790	2.161	
	$H_E$	0.619	0.711	0.802	0.725	0.199	0.611	0.239	
	$H_O$	0.375*	0.816	0.574*	0.694	0.224	0.537	0.239	
	$F_{IS}$	0.403	-0.138	0.293	0.053	-0.116	0.132	0.242	

<sup>a</sup>Markers developed by Van der Schoot et al. (2000); <sup>b</sup>Markers developed by Smulders et al. (2001).

#### AFLP genotyping

AFLP markers were generated as described in Vos et al. (1995). Total genomic DNA, isolated as described above, was double digested with *EcoRI* and *MseI* restriction enzymes and then ligated with *EcoRI* and *MseI* adapters. Pre-selective amplification was performed using a primer pair with one additional selective nucleotide, *MseI*-A/*EcoRI*-A. Selective amplifications were carried out using three different primer pair combinations: *MseI*-ACC/*EcoRI*-AAC, *MseI*-ACC/*EcoRI*-AAG and *MseI*-AGC/*EcoRI*-ATC, in which *EcoRI* primer was radiolabelled with  $\gamma$ -<sup>32</sup>P. The products of the selective amplification were electrophoretically separated on 6% denaturing polyacrylamide gels 50 cm in length (Bio-Rad Italia). Electrophoresis was conducted for 2.5 h at 110 W. AFLP fingerprints were evaluated by visual inspection of the autoradiograms. DNA bands were scored as binary characters for their presence (1) and absence (0) to produce a data matrix.

#### Data analysis

Each type of statistical analysis was carried out with the genetic marker best suited for each specific purpose. AFLPs were used, in particular, to calculate hybrid index (HI – see below), whilst SSRs were

employed to estimate all the other population genetic parameters.

#### Clonal structure

The number of genets in each population was determined using microsatellite data and the software package GenClone 1.1 (Arnaud-Haond & Belkhir 2007). In addition, this programme was used to calculate the  $P_{sex}$  index which expresses the probability that a particular genotype present more than once in the population is a result of sexual reproduction. Analysis of clonal structure was used to restrict all subsequent analyses of genetic variability to the genet level. Microsatellites were preferred over AFLPs for studying clonality and SGS analysis because of the known lower genotyping error rates associated with microsatellite loci. Nevertheless, the validity of microsatellite-based clone detection was evaluated by calculating the proportion of AFLP bands at which ramets of each microsatellite-defined genet differed from one another.

#### Genetic diversity of the sampled loci and populations

The number of alleles ( $A$ ), private and rare alleles, expected ( $H_E$ ) and observed ( $H_O$ ) heterozygosity for microsatellite loci were calculated using the GenAlEx software package (Peakall & Smouse



2006) and departures from Hardy–Weinberg equilibrium (HWE) were calculated using exact tests in GENPOP (Version 1.2 – Raymond & Rousset 1995). Subsequently, the inbreeding coefficient ( $F_{IS}$ ) and allelic richness (AR) were calculated for each population using FSTAT 2.9.3.2 (Goudet 1995). Populations were also characterised for their diversity using the dominant AFLP data and the following parameters: proportion of polymorphic bands ( $P$ ), gene diversity ( $h$ ) and Shannon diversity ( $I$ ). All these parameters were calculated by ARLEQUIN (Excoffier et al. 2005) and POPGENE (<http://www.ualberta.ca/~fyeh/>) softwares.

#### *F-statistics and migration rates*

Microsatellite-based  $F_{ST}$  between pairs of populations including jack-knifing standard errors were computed with FSTAT 2.9.3.2 (Goudet 1995). The significance of  $F_{ST}$  values was assessed by 9999 bootstrap replicates and  $P$ -values were adjusted to a nominal level of 0.05 using the Bonferroni correction (Rice 1989). The number of migrants among populations ( $N_m$ ) was calculated under the assumption of migration drift equilibrium as:  $N_m = (1 - F_{ST}) / 4F_{ST}$ .

#### *Identification of hybrids and pure *P. alba* using an AFLP-based HI*

Since riparian populations of *P. alba* are known to be highly susceptible to introgression from the related *P. tremula*, we decided to detect hybrids and introgressants using molecular markers. Maximum likelihood estimates of a HI based on dominant AFLPs were used to classify trees as either *P. alba* or *P. alba* × *P. tremula* (= *P. × canescens*) using the HINDEX programme (Buerkle 2005). The maximum likelihood HI of Buerkle (2005) was chosen over other Bayesian-based hybrid indices because it is easier to interpret. This well-established method estimates the hybridity (genomic composition) of each individual using maximum likelihood estimation based on each individual's genotype and the allele frequencies at each locus in each parental species (Buerkle 2005). The HI ranges from 0 (parental species *P. tremula*) to 1 (parental species *P. alba*). First-generation hybrids (F1s) are expected to exhibit an HI of 0.5 and first-generation backcrosses an HI of 0.25 (backcross towards *P. tremula*) or 0.75 (backcross towards *P. alba*). Individual trees were classified using arbitrary HI thresholds of 0.05 and 0.95, i.e. trees were classified as *P. alba* when  $HI > 0.95$  and as *P. × canescens* when  $0.10 < HI < 0.95$ . In order to estimate parental allele frequencies for bi-allelic AFLPs, 16 pure (non-introgressed) *P. alba* and 18 pure *P. tremula* were used. The *P. tremula* reference samples

were kindly provided by Dr Joan Cottrell (Forest Research, Northern Research Station, Roslin, Midlothian, Scotland). It would have been preferable to use Italian reference populations, but sufficient material of these was not available in the laboratory when this study was carried out. The use of north-western European material as references is justified by the fact that (1)  $F_{ST}$  of *P. tremula* at the European scale is very low ( $F_{ST} = 0.051$ ; 95% confidence interval = 0.042–0.063, Lexer & co-workers, unpublished data), and (2) most of the genetic variation in *P. alba* and *P. tremula* resides between rather than within species (Lexer et al. 2005). Reference samples of *P. alba* were from several Italian river valleys covering the area sampled in the present study. The *P. alba* reference samples were clearly identified as pure representative of the species during cutting sprouting on the basis of different bud phenology as described by Sabatti et al. (2001). These samples were kindly provided by the Dipartimento di Scienze dell'Ambiente Forestale e delle sue Risorse, Università della Tuscia, Viterbo, Italy.

#### *SGS as estimated with microsatellites*

SGS, i.e. the non-random distribution of genetic variation among individuals, was analysed for each of the three riparian populations separately at three different levels: (1) the ramet level for all sampled individuals, i.e. including *P. alba* and trees classified as *P. × canescens* based on the AFLP HI; (2) the ramet level for *P. alba* only (excluding trees classified as *P. × canescens* based on the AFLP HI); (3) the genet level for the combined *P. alba* and *P. × canescens* data, including each distinct genet of each taxon just once, using one ramet located in the centre of each genet. These three levels were chosen so as to extract a maximum of information from the data whilst at the same time minimising problems with reduction in sample size due to categorisation into species and hybrid genets. Analyses (1) and (2) provide information on changes in SGS due to the addition or removal of *P. × canescens*, whereas analysis (3) provides information on the effect of ramets on SGS. The genet level for *P. alba* was not analysed because sample sizes after removal of hybrids were too small.

To assess the effect of clonality and hybridisation on SGS, pairwise kinship co-efficients ( $F_{ij}$ ) between individuals were computed for each of the three levels of analysis, and each population and its relationship with the spatial distance separating individuals analysed, using *Spagedi* 1.2 (Hardy & Vekemans 2002). Pairwise kinship co-efficients were then regressed on the logarithm of spatial distance  $d_{ij}$  ( $d$  is the distance between  $i$  and  $j$ ) to estimate the logarithmic regression slope  $b_{log}$ . The significance of

$b_{\log}$  was tested by permuting the spatial positions of individuals 10,000 times to obtain the frequency distribution of  $b$  under the null hypothesis that  $F_{ij}$  and  $d_{ij}$  were uncorrelated (cf. Mantel test). The standard errors of  $b_{\log}$  were obtained by jack-knifing over loci. The extent of SGS was quantified using the  $S_p$  statistic following Vekemans and Hardy (2004).  $S_p$  was quantified by  $S_p = -b_{\log}/(1 - F_{(10,m)})$  where  $b_{\log}$  is the regression slope and  $F_{(10,m)}$  is the mean kinship coefficient between individuals belonging to the first distance interval (0–10 m). All these analyses were carried out on microsatellite data and were subsequently confirmed by SGS analysis based on pairwise kinship co-efficients for dominant AFLPs following Hardy (2003).

## Results

### Clone identification

The probability of encountering individuals with identical genotypes resulting from distinct sexual reproduction events with microsatellites was rather low in all populations ( $5.1 \times 10^{-10} < P_{\text{sex}} < 3.3 \times 10^{-2}$ ). Ramets of the same microsatellite-defined genet shared on average more than 93% of their AFLP bands, and this number increased to >95% when four genets carrying common microsatellite alleles were discarded. These levels of band sharing may well approach genotyping error rates encountered with manually scored AFLPs, thus microsatellite-defined genets were regarded appropriate proxies for the true clonal structure. The number of multi-ramet genets (i.e. genotypes present at least twice) detected in the studied populations was 7, 8 and 6, in Cesano, Paglia-Tevere and Ticino, respectively. The lowest  $P_{\text{sex}}$  value ( $5.1 \times 10^{-10}$ ) was estimated for a genet identified in the Cesano population, which was present six times in that population. The results allowed us to focus subsequent analyses of genetic variability and gene flow on the genet level (i.e. clonal duplicates removed).

### Characteristics of microsatellite loci in Italian populations of *P. alba*

All five nuclear microsatellites were polymorphic with up to 10 alleles observed per locus (data not shown). In total, 36 alleles were recognised in 154 individuals collected in the three populations. The number of alleles per locus in each population ranged from 1 to 7 (Table I). Two loci in Cesano and Ticino and three loci in Paglia-Tevere displayed significant departures from HWE at the genet level in the form of heterozygote deficits. One locus (WPMS15) showed consistent departures from HWE across all populations. Since null-alleles were

not previously observed in *P. alba* for the same set of SSR loci (Fossati et al. 2004; Lexer et al. 2005; van Loo et al. 2008), the heterozygote deficits may be better explained by population subdivision, inbreeding and/or varying levels of introgression. All three populations showed private and rare alleles. Cesano, Paglia-Tevere and Ticino showed one, four and six private alleles, respectively. The 199-bp (Cesano) and 229-bp (Paglia-Tevere) alleles of locus WPMS14 were the rarest with a frequency of 0.019 (Table II).

### Genetic diversity

An identical trend in the distribution of genetic diversity was found regardless of whether it was estimated using the proportion of polymorphic bands,  $h$  or Shannon diversity for 137 polymorphic AFLP bands or using allelic richness, expected ( $H_E$ ) or observed ( $H_O$ ) heterozygosity for five polymorphic microsatellites (Table III). The highest genetic diversity was found in the Ticino population followed by Paglia-Tevere and Cesano. All three populations displayed significant heterozygote deficits for microsatellites with  $F_{IS}$  ranging from 0.131 to 0.186 (Table III).

Table II. Private and rare alleles (marked with asterisks) observed for each population, and locus characterised by allele size in base pairs (bp) and frequency.

Population	Locus	Allele size (bp)	Frequency
Cesano	WPMS14	199	0.019*
Paglia-Tevere	WPMS5	313	0.041*
Paglia-Tevere	WPMS14	229	0.019*
Paglia-Tevere	WPMS15	187	0.060
Paglia-Tevere	WPMS20	228	0.028*
Ticino	WPMS14	208	0.031*
Ticino	WPMS14	232	0.112
Ticino	WPMS14	256	0.020*
Ticino	WPMS15	190	0.021*
Ticino	WPMS18	217	0.082
Ticino	WPMS18	235	0.051

Table III. Genetic diversity, at the genet level, in three introgressed riparian populations of *P. alba* in Italy, including allelic richness (AR), expected ( $H_E$ ) and observed ( $H_O$ ) heterozygosity, and inbreeding coefficient ( $F_{IS}$ ) for microsatellite markers, as well as the proportion of polymorphic bands ( $P$ ), gene diversity ( $h$ ) and Shannon diversity index ( $I$ ) for AFLPs. Departures from Hardy-Weinberg equilibrium (HWE) at  $P < 0.05$  are indicated by asterisks.

Population	Microsatellites				AFLP		
	AR	$H_E$	$H_O$	$F_{IS}$	$P$ (%)	$h$	$I$
Cesano	4.4	0.463	0.407	0.131*	39	0.155	0.239
Paglia	5.4	0.583	0.480	0.186*	65	0.154	0.247
Ticino	5.8	0.611	0.537	0.132*	72	0.211	0.327

*Genetic structure and gene flow estimated with microsatellites*

Individual  $F_{ST}$  estimates between pairs of populations were low but significant, ranging from 0.060 to 0.085 (Table IV). The highest  $F_{ST}$  value was observed between Ticino and Paglia-Tevere whilst the low  $F_{ST}$  value was found between Ticino and Cesano. The low  $F_{ST}$  values between pairs of populations translate into relatively high numbers of migrants among the populations (Table IV).

*Introgression from P. tremula*

HI estimates based on 137 polymorphic AFLP bands and a cut-off point of HI = 0.95 revealed 51 individuals (33%) with signs of introgression from *P. tremula* (= hybrid "*P. × canescens*") among the 154

Table IV. Estimates of genetic divergence  $F_{ST}$  (below diagonal ± standard errors), calculated by jack-knifing over loci and number of migrants ( $N_m$  - above diagonal) between three riverine populations of *P. alba* in Italy (Cesano, Paglia-Tevere and Ticino) based on microsatellite analysis.

	Cesano	Paglia-Tevere	Ticino
Cesano	—	3.81	3.60
Paglia-Tevere	0.060* ± 0.020	—	2.76
Ticino	0.065* ± 0.011	0.085* ± 0.049	—

\*Nominal adjusted  $P$ -value < 0.05 (Bonferroni correction).

trees analysed, comprising primarily backcrosses to *P. alba* (Figure 2). In more detail, 14, 15 and 22 genets of *P. × canescens* were identified in Cesano, Paglia and Ticino, respectively. The Ticino population not only contained the highest proportion of hybrids but also displayed the widest spectrum of HI values (Figure 2). Combined with our analysis of clonal structure (above), the HI allowed us to estimate SGS for samples of ramets or genets that either included or excluded *P. × canescens* introgressants.

*Spatial genetic structure*

Significant SGS was detected at all three levels in all three populations, except for the *P. alba* genet-level in Cesano (Table V). As expected, more structure (greater  $Sp$ ) was found at the ramet level than the genet level in all three populations (Figure 3, Table V). The slopes of kinship co-efficients ( $b_{log}$ ) at the ramet levels were clearly steeper (had higher negative values) than those at the genet level resulting in higher values for the  $Sp$  statistic (Table V). In addition, SGS analysis revealed consistently higher values for SGS at all levels in Cesano compared to Paglia-Tevere and Ticino (Figure 3).  $Sp$  values ranged from 0.0643 to 0.0331, 0.0242 to 0.0116 and 0.0183 to 0.0111 for Cesano, Paglia-Tevere and Ticino, respectively. Further, structure for ramets of *P. alba* and *P. canescens* combined (i.e. including

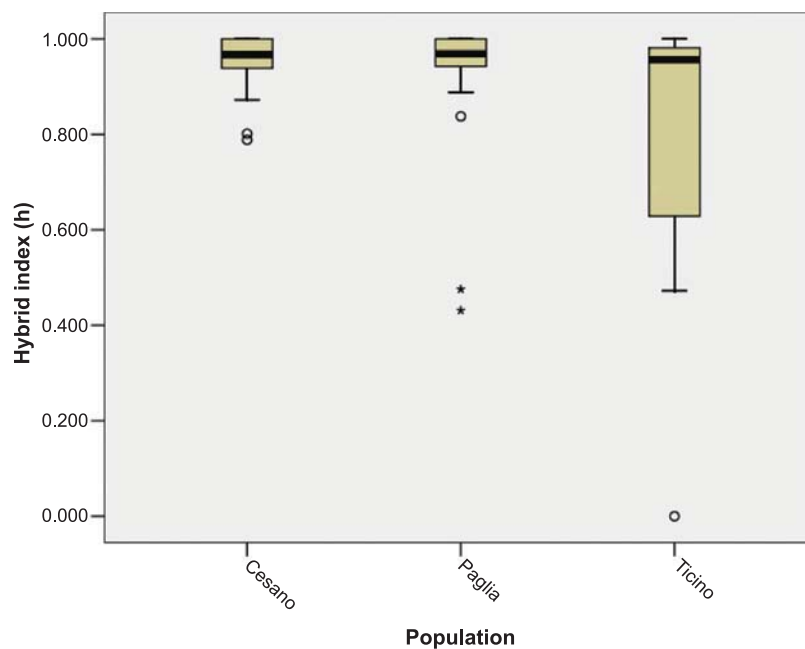


Figure 2. Maximum likelihood estimates of molecular hybrid index in three riparian populations of *P. alba* in Italy, estimated using 137 informative AFLP marker bands and parental reference samples of *P. alba* and *P. tremula* described by Fossati et al. (2004). A hybrid index of 1.000 indicates pure *P. alba*, and a hybrid index of 0.000 indicates pure *P. tremula*. The boxplots show medians, inter-quartile ranges (IQRs), outliers, extreme cases and outliers of hybrid index distributions for each population. Outliers, labelled as circles, are values more than 1.5 but less than 3 IQRs away from the end of a box. Extreme cases, labelled as stars, are values more than 3 IQRs away from the end of a box. Levels of introgression vary between the populations, including 14, 15 and 22 trees with a hybrid index < 0.950 (*P. × canescens*) in Cesano, Paglia and Ticino, respectively.

Table V. Microsatellite-based SGS analysis in three introgressed riparian populations of *P. alba* in Italy. Three sub-samples were analysed for each population. The sub-samples were defined via an AFLP-based hybrid index (HI) (*P. alba*: HI > 0.95; *P. × canescens*: HI < 0.95) and via the inclusion or exclusion of clones as identified with microsatellites. Shown are the numbers of individuals in each sub-sample (*N*), the kinship co-efficient ( $F_{ij}$ ) between pairs of trees in the focal distance class (section “Materials and methods”), regression slopes of  $F_{ij}$  on geographic distance for pairs of trees ( $b_{log}$ ) including standard errors and Vekemans and Hardy’s (2004) *Sp* statistics, which facilitates direct comparison of SGS between sub-samples and populations.

Population	Sub-sample	<i>N</i>	$F_{ij}$	$b_{log}$ (±st.e.)	<i>Sp</i>
Cesano	<i>P. alba</i> + <i>P. × canescens</i> /ramet level	52	0.2189 (±0.1471)	<b>-0.0503</b> (±0.0207)	<b>0.0643</b>
	<i>P. alba</i> /ramet level	36	0.2242 (±0.1947)	-0.0317 (±0.0295) n.s.	0.0409
Paglia	<i>P. alba</i> + <i>P. × canescens</i> /genet level	28	0.1818 (±0.2179)	<b>-0.0271</b> (±0.2126)	<b>0.0331</b>
	<i>P. alba</i> + <i>P. × canescens</i> /ramet level	53	0.2254 (±0.0417)	<b>-0.0188</b> (±0.0084)	<b>0.0242</b>
	<i>P. alba</i> /ramet level	26	0.1228 (±0.0521)	<b>-0.0142</b> (±0.0096)	<b>0.0161</b>
Ticino	<i>P. alba</i> + <i>P. × canescens</i> /genet level	36	0.1397 (±0.0604)	<b>-0.0100</b> (±0.0050)	<b>0.0116</b>
	<i>P. alba</i> + <i>P. × canescens</i> /ramet level	49	0.1704 (±0.0994)	<b>-0.0144</b> (±0.0041)	<b>0.0172</b>
	<i>P. alba</i> /ramet level	23	0.1968 (±0.0889)	<b>-0.0147</b> (±0.0096)	<b>0.0183</b>
	<i>P. alba</i> + <i>P. × canescens</i> /genet level	33	0.0971 (±0.0596)	<b>-0.0101</b> (±0.0041)	<b>0.0111</b>

Samples with significant SGS at the 0.01 level are indicated in bold. N.s., not significant.

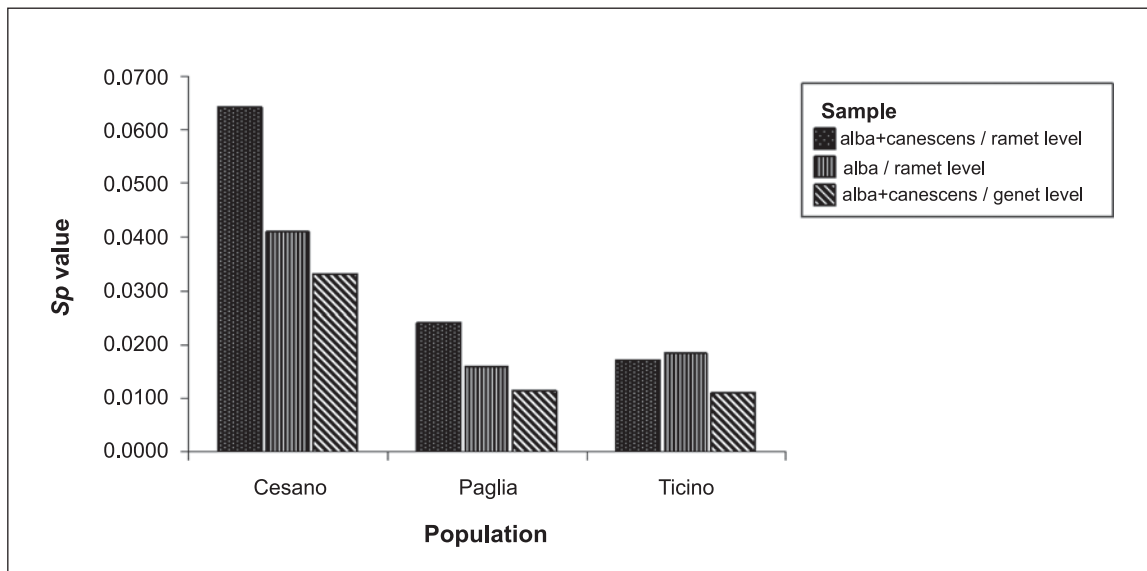


Figure 3. Spatial genetic structure (SGS) as estimated using the *Sp* statistic for microsatellites in three introgressed riverine populations of *P. alba* in Italy. The figure facilitates visual comparison of patterns of SGS across populations, and different sub-samples within populations. For definition of sub-samples, significance levels and standard errors of the SGS regression slopes see Table V. The rationale for the definition of sub-samples is outlined in the text.

introgressants) was stronger than for “pure” *P. alba* in Cesano and Paglia but not in Ticino (Figure 3). Results from complementary AFLP-based SGS analysis were largely congruent with the microsatellite-based results, indicating consistently stronger SGS for Cesano, and lower values for Paglia-Tevere and Ticino (not shown).

## Discussion

Understanding the effects of continued hybridisation on within-species genetic diversity is an important task in current conservation genetics. Hybridisation can either deplete or enrich genetic diversity, depending on factors such as the symme-

try or asymmetry of genetic barriers, effective population sizes and the fitness of hybrids (Ellstrand & Schierenbeck 2000; Barton 2001; Wolf et al. 2001; Cozzolino et al. 2006). From a pragmatic point of view, natural hybridisation can also aid conservation genetics by providing “natural mapping populations” for the identification and study of non-neutral, potentially adaptive variation, which is particularly relevant in forestry where preserving adaptive variation is recognised as an important element of forest management and conservation (Lexer et al. 2004). In European *Populus*, the conservation implications of hybridisation are manifold, as exemplified by the present study of introgressed Italian populations.



We found low levels of introgression from *P. tremula* in two of the studied populations (Cesano and Paglia-Tevere) and high levels of introgression along the Ticino river (Figure 2). Increased levels of introgression along the Ticino are accompanied by increased levels of genetic variability there, regardless of the marker type used and of the variability parameter estimated (Tables I and III). Increased introgression along the Ticino makes sense, as *P. tremula* is more frequent in the Ticino region compared to the more southernly Cesano and Paglia-Tevere river valleys; an extensive molecular genetic follow-up study of the Ticino hybrid zone revealed the frequent occurrence of pure genotypes of *P. tremula* in the southern foothills of the Alps at the Swiss/Italian border, and also in dry spots of the Ticino floodplain gallery forest (Lexer & co-workers, unpublished data).

In the smaller and more isolated Cesano and Paglia-Tevere populations, SGS increased when *P. × canescens* trees were added to the analysis (Figure 3). These observations already indicate that introgression may directly or indirectly affect genetic diversity and structure in introgressed Italian populations of *P. alba*. However, our nuclear microsatellite and AFLP data also allow us to draw information about clonality, inbreeding, patterns of diversity and gene flow, and SGS in these populations. Below, we discuss these issues with a focus on the genetic effects of introgression and implications for germplasm conservation.

#### *Clonal structure and inbreeding in introgressed Italian populations of P. alba*

Species of the genus *Populus* reproduce for the most part sexually, but also asexually through rootsuckers and cladogenesis (Dickmann 2001). Indeed, clonal spread is well documented in *Populus* (Arens et al. 1998; Winfield et al. 1998; Schweitzer et al. 2002; Barsoum et al. 2004; Storme et al. 2004; Cole 2005; Namroud et al. 2005; Suvanto & Latva-Karjanmaa 2005). Storme et al. (2004), in particular, analysing nine European germplasm banks of *P. nigra* (black poplar), reported the presence of a very high number of ramets of a single genet (78%) in the Belgian germplasm bank, made up of black poplar specimens collected in a highly managed river system. Studies of clonality in natural populations of *P. tremula*, *P. tremuloides* and *P. nigra* revealed varying levels of clonality as well (Barsoum et al. 2004; Namroud et al. 2005; Suvanto & Latva-Karjanmaa 2005). In our study, a relatively high proportion of ramets were observed (individuals present at least twice in the dataset). Similarly, a study of introgressed *P. alba* along the Danube river in central Europe revealed a comparable situation, in fact, more than 20% of ramets occurred within

multi-ramet genets (van Loo et al. 2008). Thus, our data would suggest that, in the case of *P. alba*, vegetative propagation is a commonly adopted strategy to achieve rapid colonisation after natural disturbance such as flooding. Vegetative reproduction in the populations studied here might also help to explain why we observed significant heterozygote deficits (Table I) and population-level inbreeding ( $F_{IS}$  ranging from 0.131 to 0.186;  $P < 0.05$ ; Table III). A high number of ramets per genet will result in a large amount of viable pollen and seeds, thus increasing the chances of successful establishment and survival of progeny derived from that genet. Moreover, the presence of several ramets in a certain area will effectively increase the chance to intercept viable and compatible pollen, as demonstrated by Vanden Broeck et al. (2006) in the case of *P. nigra*. Thus, it is likely that clonality will contribute to localised departures from random mating in populations of *P. alba*.

The  $F_{IS}$  values observed here were lower in the Ticino and Cesano populations (0.132 and 0.131, respectively) compared to Paglia-Tevere (0.182). Thus, we propose, with caution, that the increased  $F_{IS}$  in Paglia-Tevere could also be ascribed to the greater patchiness and greater levels of disturbance in this drainage system. The Paglia-Tevere river, in fact, runs between agricultural fields, its naturalness often compromised by human activities and *P. alba* grows in a narrow stripe along the banks of the river. The Cesano and Ticino populations have experienced less human interference than the Paglia-Tevere. The Ticino river population, in particular, is part of a well-preserved floodplain forest in a Regional Park founded in 1974. Here, *P. alba* grows in numerous stands along the river, embedded within the floodplain forest in a “mosaic-like” manner. Considering high levels of gene flow (Table IV; Lexer et al. 2005) and the considerable gene dispersal distances typical of floodplain populations of *P. alba* (sigma up to 280 m; van Loo et al. 2008), it is not surprising that the Ticino population deviates less strongly from random mating than Paglia-Tevere.

#### *Patterns of genetic diversity and gene flow in introgressed Italian populations of P. alba*

Despite some degree of inter-locus variation (Table I), genetic diversity was on average higher along the Ticino compared to the other populations (Table III). Cesano is always the population showing the lowest values for these parameters. This repetitive trend probably is due to two main factors. First, the Ticino population is the largest population and it is situated in an unmanaged floodplain forest comprising hundreds of square kilometres. Second, the

Ticino population also presents the largest number of hybrids and introgressants (45%, Figure 2). Consequently, diversity in the Ticino population is enriched by the large proportion of alleles introgressed from *P. tremula* (Figure 2).

The heterozygosities ( $H_O$ ) estimated here (Tables I and III) indicate relatively high levels of genetic diversity in Italian populations of *P. alba*, comparable with previous microsatellite-based estimates for populations of *P. alba* (0.419 and 0.341) and *P. tremula* (0.466 and 0.483) along the Danube (Lexer et al. 2005) and comparable also with the North American *P. tremuloides* (0.460, 0.310, 0.560 and 0.410) (Cole 2005). Thus, Italian populations of *P. alba*, despite evident inbreeding and landscape fragmentation, appear to retain levels of neutral diversity similar to other natural populations of poplars and aspens.

The low  $F_{ST}$  values observed among the studied populations (0.060–0.085) were as expected for this wind-pollinated, wind-dispersed species (Lexer et al. 2005). Less expected was the low level of divergence ( $F_{ST}$ ) for Cesano and Ticino, as these two populations are separated by a much larger geographic distance than the neighbouring Paglia-Tevere and Cesano (Figure 1). This would suggest a role for the Apennine mountain range to act as a barrier to gene flow between these adjacent populations of *P. alba*, even if this preliminary observation should be supported by the analysis of additional populations of *P. alba*. More generally, divergence ( $F_{ST}$ ) between pairs of Italian populations of *P. alba* is similar to that calculated for *P. alba* along the Danube (Lexer et al. 2005) and slightly greater than that estimated for North American aspen by Wyman et al. (2003) and Cole (2005). Even if Italian populations of *P. alba* show several private and rare alleles (Table II), the number of migrants between populations (Table IV) is appreciably high ( $N_{em} = 3\text{--}4$  migrants per generation). Thus, taken together, the data would suggest that the three studied Italian populations of *P. alba* are part of a well-connected meta-population system with occasional genetic discontinuities introduced by geographic barriers.

#### *Spatial genetic structure*

Analysis of SGS was possible at the ramet level for *P. alba* and *P. × canescens* combined, at the ramet level for *P. alba* only, and at the genet level for *P. alba* and *P. × canescens* combined (Figure 3; Table V). Although an analysis at the genet level for pure *P. alba* was not possible due to limitations in sample size, the available data allowed us to assess between-population variation in SGS and the effects of clonality and introgression on SGS. The latter topic (multi-locus SGS in natural introgressed

populations) has very rarely been discussed in the literature (see Cornman et al. 2004; Valbuena-Carabana et al. 2007; van Loo et al. 2008).

Perhaps, the most conspicuous pattern emerging from the data is that of a large between-population variation in the absolute strength of SGS (Figure 3). SGS in plant populations is affected by a multitude of demographic and ecological factors (Vekemans & Hardy 2004; Valbuena-Carabana et al. 2007), so these differences may not be surprising. At the very least, our results confirm the importance of population replicates in studies of SGS. Nevertheless, we are interested in patterns of SGS among different subpopulations within each locality, rather than in absolute differences between localities.

As expected, SGS in each locality was generally weaker at the genet level (clones removed), thus demonstrating the effect of clonality on SGS (Figure 3). In Cesano and Paglia-Tevere, SGS was also weaker in *P. alba* at the ramet level than in *P. alba + P. canescens* at the ramet level (Figure 3), thus suggesting direct or indirect effects of introgression on SGS. Stronger SGS in introgressed relative to pure *P. alba* has been previously shown for a large introgressed population of *P. alba* along the Danube in central Europe (van Loo et al. 2008). Interestingly, this trend was not observed for Ticino, the population with the highest levels of introgression (Figure 2), where SGS remained unchanged when introgressed *P. canescens* were added to the analysis (Figure 3; standard errors of  $b_{log}$  overlap, see Table V). This may be better explained by recent observations on natural hybrids between the European oaks *Quercus pyrenaica* and *Quercus petraea* (Valbuena-Carabana et al. 2007). There, hybridisation did not result in an increase of SGS, rather, it tended to dilute the SGS present in the parental species. One possibility to explain the differences between SGS in introgressed *P. alba* in the Ticino population compared to the Danube population (van Loo et al. 2008) is that, along the Ticino river, introgression may have started earlier. This may have allowed sufficient time for equilibrium to arise, i.e. SGS induced by the localised introduction and spread of “foreign” *P. tremula* alleles may have already disappeared. We note that the Ticino hybrid zone is located at the south side of the Alps where secondary contact between *P. alba* and *P. tremula* may have occurred earlier than along the Danube at the north-eastern edge of the Alps. An alternative explanation may be that assortative mating in *P. × canescens* introgressants is more pronounced along the Danube (van Loo et al. 2008). Our results indicate the need for more extensive sampling of the Ticino population to test these hypotheses.

### *Implications for the conservation of neutral and non-neutral diversity*

Combined with results obtained for the Danube river valley (Lexer et al. 2005), Hungarian drainage systems (Bartha 1991) and other European river valleys (Rajora & Dancik 1992), our present study of Italian populations indicates that hybridisation and introgression from *P. tremula* into *P. alba* are widespread across Europe although levels of introgression vary greatly among localities. The fact that effective population sizes in *P. alba* appear to be smaller than in *P. tremula* (Lexer et al. 2005), and that Italian populations of *P. alba* are often confined to thin and patchy gallery forests suggests that introgression may potentially pose a threat to *P. alba*, i.e. the species may be swamped by gene flow from *P. tremula*. However, gene flow from *P. tremula* also enriches local gene pools of *P. alba* as shown here for the Ticino population, and by Lexer et al. (2005) for the Danube valley population of this species. The observation of broad and rather continuous genotypic distributions in hybrid populations (Figure 2; Lexer et al. 2005) suggests that isolating barriers are incomplete, and that out-breeding depression is not strong enough to prohibit introgression. If so, then introgression from *P. tremula* may potentially allow *P. alba* to colonise the upstream portions of river valleys, which may otherwise be too cold for this warmth-loving species. This would imply a potential for adaptive introgression, which is a very little explored topic (see Rieseberg et al. 1999; Barton 2001; Martin et al. 2006). Data on the fitness of phenotypes and genotypes in hybrid populations will be necessary to test these hypotheses.

Considering the genetic data shown here and results of previous studies of introgressed *P. alba* (Bartha 1991; Rajora & Dancik 1992; Lexer et al. 2005), we suggest that the main conservation implication of our data refers to the potential for the identification and study of non-neutral DNA variation. Present data indicate that the Ticino hybrid population represents a valuable “replicate” population for population genomic work aimed at identifying genome regions under selection (Lexer et al. 2007). Hybrid zones between *P. alba* and *P. tremula* have been identified as being suitable for the detection of locus-specific effects due to intrinsic and ecological selection pressures, and for the genetic mapping of phenotypic traits using admixture LD (Lexer et al. 2007). Since the two hybridising species are ecologically divergent (Adler et al. 1994), it is expected that some of the non-neutral DNA variation identified in natural hybrid populations will point to loci, or genomic regions subject to ecological selection. The Ticino population contains

a high proportion of hybrids (45% of sampled trees), most of which appear to be recombinant backcrosses (Figure 2), and patterns of SGS (Figure 3) suggest that inter-specific gene flow has been in progress for a long time. Thus, this population should be suitable for addressing one of the most important topics in current forest conservation genetics, namely the detection of non-neutral, potentially adaptive variation for germplasm conservation, breeding and restoration ecology.

### **Acknowledgements**

We are grateful to Joan Cottrell for providing reference samples of *P. tremula* and to Berthold Heinze for helpful and stimulating discussions. S. Castiglione’s research was supported by the Italian Ministry of Environment, Land and Sea Protection (Ministero dell’Ambiente e della Tutela del Territorio e del Mare) within the research project “Research and development in biotechnology applied to the protection of the environment”, in collaboration with The People’s Republic of China. C. Lexer’s research on within-species variation for genomic isolation in European *Populus* is supported by grant NE/E016731/1 of the Natural Environment Research Council (NERC) in the UK.

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